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SITE-SPECIFIC DIFFERENTIATION OF FIBROBLASTS IN NORMAL AND SCLERODERMA SKIN

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14. ABSTRACT

The results from year 2 of funding applied novel biomarkers to address whether systemic sclerosis, a fibrosing disorder, involves changes in cell positional identity. Long noncoding RNAs are the predominant position specific output of the HOX loci, and their misexpression in systemic sclerosis suggests that altered fibroblast positional identity is involved.

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INTRODUCTION:

In systemic sclerosis (SSc), fibrosis of skin and internal organs occurs in an anatomically reproducible and progressive fashion. The extent and localization of skin involvement are important predictors of long-term outcome and mortality, but the basis of site specificity in SSc is not understood. Fibroblasts are the principal cells in dermis and stroma of epithelial organs that synthesize extracellular matrix proteins, and they are believed to be responsible for excessive fibrosis and tissue hardening in SSc. We recently discovered that fibroblasts are systematically differentiated in a site-specific manner, and they vary significantly in the expression of many genes related to extracellular matrix synthesis and turnover. We hypothesize that the site-specific differentiation of fibroblasts plays an important role in the anatomic specificity of SSc, addressed in three specific aims. At the end of the funding period, we will have characterized fibroblast gene expression patterns in both normal and scleroderma skin and identified the specific fibroblast populations and their gene products that might be associated with SSc progression. This new knowledge will provide basic and much-needed insights on fibroblasts and the pathogenesis of SSc.

Specific Aim 1: Profile fibroblast populations from skin of diverse anatomic sites, thereby creating a reference database of markers for site-specific fibroblast populations.

Specific Aim 2: Construct tissue microarrays of skin and scleroderma tissues to enable high throughput visualization of fibroblast gene and protein expression.

Specific Aim 3: Quantify site-specific populations of fibroblasts in SSc and compare them with site-matched normal and control skin tissues

BODY:

To test the hypothesis that altered fibroblast positional identity may relate to SSc pathogenesis, our first task is to identify reliable markers of fibroblast positional identity in normal skin tissues. In year one of this project, we identified a novel type of genes, termed long noncoding RNAs (lncRNAs), as a major type of transcripts that are expressed in an anatomic specific manner in the body (Rinn et al., 2007). Long noncoding RNAs are transcribed by RNA polymerase II, capped, spliced, and polyadenylated, but do not function by serving as the instruction for protein synthesis. Instead, we and others have found that lncRNAs may serve as the interface between DNA and chromatin modification activities (Amaral et al., 2008; Kapranov et al., 2007; Rinn et al., 2007). The HOX loci encode the master regulators of positional identity in animal cells; the HOX transcription factors are expressed in a nested fashion along the anterior-posterior axis and proximal-distal axis {Wang, 2009 #1401}. We recently characterize the transcriptional landscape of the four human HOX loci at five base pair resolution in 11 anatomic sites {Rinn, 2007 #1166}. We identified 231 HOX ncRNAs that extend known transcribed regions by more than 30 kilobases. HOX ncRNAs are spatially expressed along developmental axes and possess unique sequence motifs, and their expression demarcates broad chromosomal domains of differential histone methylation and RNA polymerase accessibility. Thus, HOX lncRNAs are the predominant position specific output of the HOX loci, and may be ideal biomarkers to address whether positional identity is altered in SSc.

In year two of the project, we have now addressed whether HOX lncRNAs may be misexpressed in SSc lesions. We employed a custom-designed, ultra-high resolution tiling array that interrogated the human HOX loci at five-base resolution. We hybridized RNA from site-matched normal skin and lesions of SSc, all derived from the forearm (n=5). We found that nine HOX lncRNAs are consistently dysregulated in SSc skin compared to control skin. Eight of these RNAs are increased in expression; one is decreased in expression. A majority of the lncRNAs increased in expression are those normally expressed in a distal or posterior anatomic fashion. Thus, this preliminary data suggests that accentuation of the distal or posterior fate is associated with SSc. Interestingly, distal anatomic sites, such as fingers, are frequently the initial sites of disease of SSc.

In the second aim of the proposal, we proposed to create tissue microarrays of skin in order to facilitate high throughput interrogation of RNA or protein expression. Gene and tiling microarrays are useful for elucidating the genetic and epigenetic elements that differentiate cell types across the body; however it is equally important to determine the *in vivo* anatomic localization of genes in the three-dimensional context of the skin. To address this challenge, we have constructed a “skin diversity” tissue microarray, where multiple skin sections are placed on a single slide to be used for in situ hybridization and immunohistochemistry. Our tissue microarrays is comprised of 42, two-millimeter formalin-fixed, paraffin-embedded cores of skin from diverse anatomic sites and 8 internal organs such as cervix, intestine, lung, liver and bone (Rinn et al., 2008). Immunohistochemistry or RNA in situ hybridization can be performed on all 50 tissues in parallel, allowing unbiased and high throughput comparison of protein or gene expression levels and localization. A potential limitation of this technology is that proteins and mRNAs present in low levels may be better visualized in frozen sections than formalin-fixed tissues, and conditions for antigen retrieval or signal amplification may need to be developed to visualize low abundance gene products.

To illustrate the use of such a skin diversity tissue microarray, we performed RNA in situ hybridizations for Keratin 14 (K14) and Keratin 9 (K9). As expected, we observed expression of K14 in the basal layer of epidermis in skin from all anatomic sites. K9 is a suprabasal keratin of plamo-plantar skin, and indeed only palmo-plantar skin on our tissue microarray showed strong K9 signal (Rinn et al., 2008). The skin diversity tissue microarray should be useful for the discovery and validation of novel site-specific genes or signaling pathways. Again, this approach can be extended to include tissues of skin diseases to monitor the in vivo expression of genes perturb in disease. The combined power of gene expression, tiling and tissue microarrays will greatly facilitate our understanding of the genes that are important in skin patterning and their roles in skin disease.

KEY RESEARCH ACCOMPLISHMENTS:

- Identification of new position-specific gene markers, including noncoding RNAs
- Interrogation and identification of altered noncoding RNA expression in scleroderma
- Construction of skin tissue microarrays
- Use of skin diversity TMA to interrogate site-specific gene expression

REPORTABLE OUTCOMES:

One publication, listed below.

1. Rinn, J. L., Wang, J. K., Liu, H., Montgomery, K., van de Rijn, M., and Chang, H. Y. (2008). A systems biology approach to anatomic diversity of skin. J Invest Dermatol *128*, 776-782.

CONCLUSION:

These results suggest that one part of the perturbation in scleroderma fibroblasts involves altered positional identity. This alteration is specifically manifested in misexpression of long noncoding RNAs from the HOX loci. Additional samples are necessary to confirm and extend these results.

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Rinn, J.L., Wang, J.K., Liu, H., Montgomery, K., van de Rijn, M., and Chang, H.Y. (2008). A systems biology approach to anatomic diversity of skin. J Invest Dermatol *128*, 776-782.

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Rinn, J. L., Wang, J. K., Liu, H., Montgomery, K., van de Rijn, M., and Chang, H. Y. (2008). A systems biology approach to anatomic diversity of skin. J Invest Dermatol *128*, 776-782.

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Montagna Symposium on the Biology of Skin

A Systems Biology Approach to Anatomic Diversity of Skin

John L. Rinn[1], Jordon K. Wang[1], Helen Liu[1], Kelli Montgomery[2], Matt van de Rijn[2] and Howard Y. Chang[1]

Human skin exhibits exquisite site-specific morphologies and functions. How are these site-specific differences specified during development, maintained in adult homeostasis, and potentially perturbed by disease processes? Here, we review progress in understanding the anatomic patterning of fibroblasts, a major constituent cell type of the dermis and key participant in epithelial–mesenchymal interactions. The gene expression programs of human fibroblasts largely reflect the superimposition of three gene expression profiles that demarcate the fibroblast's position relative to three developmental axes. The HOX family of homeodomain transcription factors is implicated in specifying site-specific transcriptional programs. The use of gene, tiling, and tissue microarrays together gives a comprehensive view of the gene regulation involved in patterning the skin.

Journal of Investigative Dermatology (2008) **128,** 776–782; doi:10.1038/sj.jid.5700986

Introduction

The human skin shows remarkable diversity in its structure and function across anatomic sites. Scalp skin is easily recognizable by the numerous terminal hair follicles; in contrast palmoplantar skin possesses no hair follicles but is characterized by increased number of eccrine glands and compact hyperkeratosis of the stratum corneum. The anatomic diversity of human skin is also reflected in the site specificity of many skin diseases and their response to treatment; the distribution of skin lesions is indeed often one of the key clues to the correct diagnosis. From a basic science perspective, the anatomic diversity of skin raises many intriguing questions about how cells acquire and maintain their positional identities in a complex, self-renewing tissue. Here, we review recent progress in understanding site-specific differentiation of cell types in the skin, focusing on emerging systems biology approaches that are beginning to provide comprehensive descriptions of this fascinating biology.

Studies of skin development have shown that site-specific differentiation of epithelia critically depends on epithelial–mesenchymal interactions. Hair and other skin appendages develop through a complex series of reciprocal interactions between epidermal cells and fibroblasts, beginning with a signal from dermal fibroblasts to the overlying epidermis to proliferate, forming the placodes that are progenitors of hair follicles (Millar, 2002). Classic heterotopic recombination

Editor's Note

In 1950, Dr William Montagna, a biologist at Brown University, began a symposium that was designed to bring together basic scientists and clinically trained dermatologists to further our understanding of skin (see Origin of the annual symposium on the biology of skin. Kligman AM (2002) *J Invest Dermatol Symp Proc* 7:1). This month we celebrate the 56th Annual Montagna Symposium on the Biology of Skin, which focused on the development and diseases of skin appendages, with two Perspectives articles. Dr Montagna would be pleased with such a topic, as much of his investigative interest was focused on skin appendages, including hair and sebaceous glands, and on the development of skin, from mice to primates. In the first article, Rinn and co-authors highlight how the emerging science of systems biology can be used to study and understand the wide diversity that is present in skin. In the second article, Wang and co-authors describe how signaling in the skin through Smads can impact skin appendage development. These articles highlight how the application of new technology and new signaling pathways can be applied to skin biology to help us understand the complexity of skin.

Russell P. Hall III,
Deputy Editor

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Abbreviations: EC, endothelial cell; nt, nucleotide

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experiments showed that a primary dermal signal dictates the overlying epithelial fate; for instance, grafting of wing epidermis to foot dermis in chick led to the development of scales rather than feathers (Dhouailly, 1973, 1984). Specialized fibroblasts from the dermal papilla of hair follicles (Jahoda *et al.*, 1984), but not dermal fibroblasts a few hundred microns away, are able to induce *de novo* hair follicle development when transplanted into naïve skin with epidermal stem cells (Blanpain *et al.*, 2004). Moreover, the type of hair varies throughout the body, and this positional information also is dictated by the local fibroblasts. For example, dermal papilla fibroblasts derived from whiskers induce long, thick whisker-like hairs when transplanted into recipient animals at heterotopic sites (Jahoda, 1992). These intimate and specific epithelial–mesenchymal interactions are not confined only to skin, but appear to be a major theme in the development and homeostasis of all epithelial organs: local fibroblast-like cells in the urogenital sinus induce differentiation of prostatic epithelial precursors to form the prostate gland (Cunha, 1994), and local fibroblasts are also responsible for metanephric induction and pruning of nephrons in the kidney (Schedl and Hastie, 2000; Levinson and Mendelsohn, 2003). Branching morphogenesis of the lung similarly depends critically on reciprocal interactions between bud epithelial cells and surrounding fibroblasts (Shannon and Hyatt, 2004). Because the epidermis is continually shed and replaced by newly developed keratinocytes (every 28 days in humans), it stands to reason that the site-specific inductive capacity of fibroblasts must persist into adulthood, perhaps through the entire lifetime. For instance, cell–cell contact between adult palmoplantar fibroblasts with trunk keratinocytes reprograms these keratinocytes to express palmoplantar keratin genes (Yamaguchi *et al.*, 1999).

Fibroblasts are the principal cell type in the dermis and stroma of internal organs that synthesize connective tissue proteins such as collagen (Dunphy, 1963; Gabbiani and Rungger-Brindle, 1981). Fibroblasts were first described histologically in 1847 by Schwann as ''spindle-shaped or longish corpuscles which are thickest in the middle and gradually elongated in both extremities into minute fibres'' (Schwann, 1847). In practice, fibroblasts are usually identified by their spindle-shaped morphology, the ability to adhere to plastic tissue culture vessels, and the absence of markers for other cell lineages (Dunphy, 1963; Gabbiani and Rungger-Brindle, 1981; Normand and Karasek, 1995). Given these rather non-selective criteria, it is not surprising that cells we currently consider ''fibroblasts'' may comprise a diverse group of cells, each with distinct patterns of synthetic activities and functions.

Site-specific differentiation of fibroblasts

A first test of this idea came with the comprehensive gene expression analysis of primary human dermal fibroblasts from distinct anatomic sites (Chang *et al.*, 2002). Using cDNA array technology, we analyzed the expression of ~21,000 genes in 50 primary human fibroblasts culture from 10 anatomic sites. The results revealed that although all fibroblasts are morphologically similar, the gene expression patterns of cultured fibroblasts from different sites are strikingly distinct. Approximately 8% of all genes transcribed in fibroblasts are differentially expressed in a site-specific manner. The variation and magnitude of gene expression differences among fibroblasts are comparable to the variation observed among different types of white blood cells. When their gene expression patterns were grouped by similarity using a technique called hierarchical clustering (Eisen *et al.*, 1998), fibroblasts from the same topographic sites of the skin were consistently grouped together, and the distinctiveness of topographic gene expression was not obscured among different donors, by passage in tissue culture, or by environmental changes such as serum starvation. In four cases where fibroblasts were derived from multiple sites of the same individual, fibroblasts from the same site of different individuals were far more similar to each other than fibroblasts from different sites of the same individual. These results demonstrate that in fact there are many different cell types that go under the traditional heading of ''fibroblasts''. The main rule of differentiation among dermal fibroblasts appears to be dictated by the anatomic site of origin.

The genes expressed by fibroblasts in a site-specific manner demonstrate distinct choreographed programs in extracellular matrix synthesis, lipid metabolism, and signaling pathways controlling cell migration and cell fate specification (Chang *et al.*, 2002). The gene expression patterns particular to distinct types of fibroblasts were biologically consistent with their anatomical origin. The site-specific expression of many cell growth and differentiation molecules such as members of TGF-β, Wnt, receptor tyrosine kinase and phosphatase families indicates that fibroblasts make important contributions in mesenchymal induction of epithelia. Moreover, the expression domains of genes underlying several genetic diseases affecting skin or musculoskeletal connective tissue correlated closely with the phenotypic defects. For example, by comparing genes that were induced in dermal fibroblasts compared to lung fibroblasts, we were able to identify the genes involved in 6 out of 10 types of Ehlers–Danlos syndrome, a congenital disease characterized by skin fragility and joint laxity. Similarly, we observed that HOXA13 is induced in toe and foreskin fibroblasts, and mutation of HOXA13 in humans leads to hand–foot–genital syndrome, a disease characterized by syndactyly, hypospadias, and malformations of the urogenital system. These results indicate that many important aspects of site-specific differentiation in fibroblasts are preserved *in vitro* and thus amenable to dissection by molecular and functional genomic approaches.

Many epithelial–mesenchymal interactions that specify epidermal appendages occur within local signaling environments of even finer specialization of fibroblasts. Overlaid on the regional fibroblast specialization, recent genomic profiling experiments have indeed identified gene expression signatures of such specialized cells, such as that of the

dermal papilla cells (Rendl *et al.*, 2005). Similarly, we and others have identified unique gene expression signatures of stromal fibroblasts of basal-cell carcinomas, a cancer of hair follicle origin, that distinguished such cells from local fibroblasts of the face (Sneddon *et al.*, 2006).

In contrast, genome-wide expression profiling of purified endothelial cells (ECs) revealed that unlike fibroblasts, EC differentiation is primarily dictated by the vessel size of origin. ECs derived from large vessels from various anatomic locations are more similar to each other than ECs from microvasculature. In particular, dermal microvascular ECs from different anatomic sites were not particularly distinct and were similar to microvascular ECs from intestine and lung (Chi *et al.*, 2003). These results reveal the contrasting regulatory logic of different stromal cell types and reinforce the uniqueness and likely developmental importance of the site-specific differentiation of fibroblasts.

Patterning of adult fibroblasts along three developmental axes

In his influential treatise on development Lewis Wolpert (1969), famously postulated two potential mechanisms of achieving pattern formation: spatial organization of cellular differentiation may be achieved by unique specification of each cell type; alternatively, organization may arise by cells interpreting their position relative to reference points, and adopting specific differentiation programs based on their positional identity within a coordinate system. A particularly attractive and distinctive feature of the positional identity model is the parsimonious use of molecular entities to construct the system, leading to universality of the coordinate system. For example, the same reference points can be used to specify the proximal–distal axis of the upper and lower limbs even though the limbs are spatially distant from each other. Similarly, the same reference points can pattern the anterior–posterior axis of the trunk and also distinguish the upper and the lower limbs.

We hypothesized that these models of pattern formation can be distinguished by comparing the global gene expression profiles of cells distributed throughout the body. Pattern formation by unique specification predicts that the similarity of gene expression profiles of cells would be sporadic and not related by distance from their sites of origin. Conversely, if the positional model of specification is at work, expression profiles will exhibit spatial relationships between fibroblasts in similar positional quadrants of the body.

To distinguish between these models, we analyzed the genome-wide gene expression profiles of 47 primary fibroblast cultures from 43 unique anatomical sites spanning the entire human body (Figure 1a) (Rinn *et al.*, 2006). We confirmed at a much finer level that fibroblasts exhibited large-scale differences in their gene expression programs in a site-specific fashion. Moreover, comparison of gene expression differences among cells from local vicinities *versus* those far away revealed that these gene expression differences are globally related to three recurring segmental patterns: proximal–distal, anterior–posterior (rostral–caudal), and internal–external (non-dermal *vs* dermal) (Figure 1b). For instance, fibroblasts originating from distal upper limb (hand, fingers) had a distinct gene expression signature compared to those from more proximal sites of the arm; fibroblasts from the leg also showed distinct gene expression signatures that portioned them to those above and below the ankle. Remarkably, comparison of the distal arm and distal leg gene signatures showed that these signatures were largely the same, suggesting that this was a signature that reflected distal position along a developmental axis, regardless of the actual position on upper or lower limb. Similarly, the gene expression data that automatically distinguished fibroblasts originating from the top half of the body (anterior or rostral) had a distinctive gene expression profile than from those from the bottom half of the body (posterior or caudal). A third gene expression signature distinguished dermal fibroblasts from fibroblasts that originate from internal organs. These findings suggest

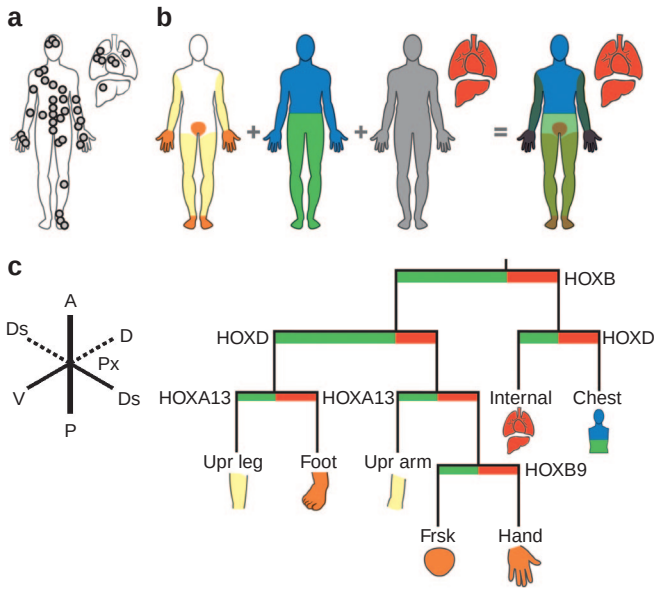

Figure 1. The embryonic pattern of HOX gene expression is retained in adult human fibroblasts. (**a**) A total of 47 primary cultures representing 43 unique anatomic sites (gray circles) that finely map the human body were profiled by gene expression microarrays. (**b**) Model of fibroblast differentiation by overlapping positional patterns of gene expression: proximal (yellow), distal (orange), anterior (blue), posterior (green) and internal organs (red). (**c**) A decision tree of HOX expression that distinguish unique anatomic positions. Right: red indicates higher-than-average and green lower-than-average expression of a given HOX gene, relative to the average expression level across 47 cultures. Each anatomic site can be correctly identified by monitoring the expression level of three HOX genes or less. Left: the developmental axes that demarcate site-specific gene expression of fibroblasts: **a**nterior–**p**osterior, **p**roximal–**d**istal and dermal–non-dermal. A third developmental axis, **d**orsal–**v**entral, did not correlate with large-scale site-specific gene expression in fibroblasts.

that site-specific variations in fibroblast gene expression programs are not idiosyncratic, but rather are systematically related to their positional identities relative to major anatomic axes. Much like a global positioning system, the site-specific gene expression program of a fibroblast reflects the superimposition of three positional coordinates, potentially providing critical cues for development, trafficking, and homeostasis of surrounding cells in the skin.

The positional identities of adult fibroblasts raise the question of whether their cognate coordinate system was established during embryonic development. During embryogenesis, expression of specific HOX genes demarcates distinct positional identities that lead to site-specific cellular differentiation and tissue morphogenesis. The HOX family of homeodomain transcription factors comprise 39 genes that are clustered on four chromosomal loci; their physical order on the chromosomes reflects their spatial pattern of expression along the anterior–posterior and proximal–distal axes of the embryo—a property called colinearity. We found that a very similar pattern of HOX gene expression was also retained in fibroblasts both *in vitro* and *in vivo* (Rinn *et al.*, 2006). In fact, HOX gene expression alone was predictive of the anatomic origin of a given fibroblast culture (Figure 1c). For example, both dermal and non-dermal fibroblasts from the trunk express HOXB genes. However, HOXD gene expression is limited to dermal fibroblasts and is not found in non-dermal fibroblasts. Moreover, distal fibroblasts can be distinguished from proximal fibroblasts by HOXA13 gene expression. Thus, using a very simple decision tree of HOX gene expression, the anatomic position of a fibroblast can be predicted (Figure 1c). HOX genes may also be globally expressed in skin in select circumstances, such as the HOXC13 gene during terminal hair follicle development (Godwin and Capecchi, 1998); however, we mainly observed canonical, site-specific expression of HOX genes in fibroblasts. The salient description of fibroblast anatomic origin by HOX gene expression suggests that this family of transcription factors may have a role in the establishment of site-specific fibroblast gene expression programs.

Revealing the epigenetic landscape by tiling microarrays

Major features of the embryonic pattern of HOX gene expression are retained in adult human fibroblasts from young and old donors alike, and these site-specific patterns of HOX gene expression are also preserved after extensive *ex vivo* cell divisions (J.L.R. and H.Y.C., unpublished observation). The stability of the site-specific transcriptional patterns suggests the possibility of epigenetic mechanisms in their maintenance. Classic genetic studies in *Drosophila* established that HOX gene expression patterns are maintained by two opposing histone modification activities. The Polycomb group proteins mediate histone H3 lysine 27 methylation and are required for the maintenance of HOX gene repression, whereas the Trithorax proteins mediate histone H3 lysine 4 methylation and are required for maintenance of HOX gene activation (Ringrose and Paro, 2004). In addition, intergenic transcription of long noncoding RNAs also plays important roles in the epigenetic maintenance of HOX transcriptional patterns (Schmitt and Paro, 2006). While some of these components are known in model organisms, the transcriptional and epigenetic landscapes in human cells are much less understood. A comprehensive view of transcription and chromatin structure of the HOX loci is needed to decipher the regulatory mechanisms that result in the spatial and temporal patterning of HOX gene expression.

Primary cells from distinct anatomic sites of human skin offer a unique resource of purified cells of specific positional identities that may be used to interrogate the epigenetic mechanisms of site-specific HOX expression programs. We and others have used a technology called tiling microarrays to gain a comprehensive view of the HOX loci (Bernstein *et al.*, 2005; Guenther *et al.*, 2005; Squazzo *et al.*, 2006) (Figure 2). Unlike conventional DNA

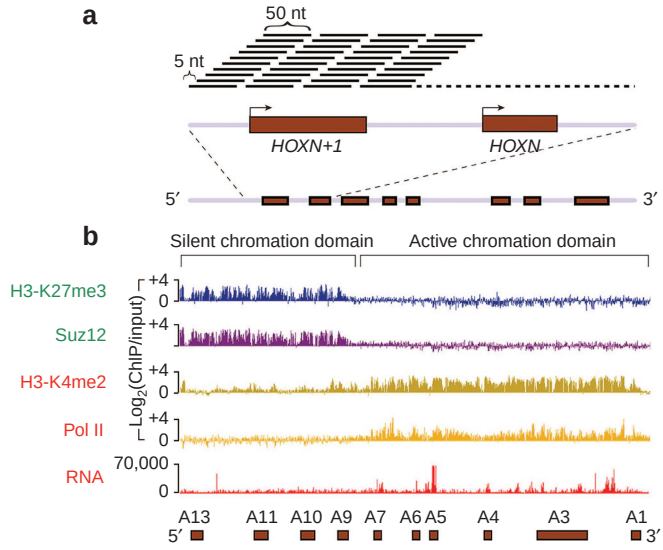

Figure 2. The epigenetic regulation of HOX expression patterns in fibroblasts. (**a**) Schematic representation of the DNA tiling design consisting of ~400,000 fifty-nucleotide probes that overlap by 45 nt such that each probe interrogates a unique 5 nt sequence. The tiling array covers all four human HOX loci and 2 Mb of control regions. Brown boxes represent specific HOX genes. (**b**) Opposing histone modifications demarcate transcriptional accessibility of HOXA locus. The top four rows show occupancy for the named protein (polycomb group protein Suz12 or RNA polymerase II (PolII)) or histone modification (H3K27me3 and H3K4me2) across ~100 kb of the HOXA locus (*x* axis), as measured by ChIP-chip. The *y* axis plots the ratio of hybridization signals of chromatin immunoprecipitation over input genomic DNA in log 2 space. The bottom row shows RNA hybridization signal over the same genomic region in linear scale (0–70,000 intensity units). Chromatin immunoprecipitation experiments were performed as described (Squazzo *et al.*, 2006).

microarrays where one probe is placed for each gene of interest, the tiling microarray employs many more probes to tile across genomic regions of interest with large overlap between adjacent probes (Figure 2a). We constructed a HOX tiling array that interrogates all four human HOX loci (HOXA, B, C, D) using 50-mer oligonucleotide probes that overlap by 45 nucleotides (nt). Thus, each 5 nt of DNA sequence is uniquely interrogated. Hybridization of RNA or chromatin immunoprecipitation on tiling microarrays (so-called ChIP-chip analysis) then reveals all of the transcribe regions or sites of chromatin modification(s) in an unbiased manner. Conventional ChIP experiments typically yield DNA fragments of 500–1,000 base pairs (bp) and hence can be interrogated by much lower resolution promoter or tiling arrays. However, interrogation of nucleosome positioning (~140 bp per nucleosome) and novel noncoding RNA (including small RNAs < 100 nt) require substantially higher resolution for their demarcation.

We and others have found evidence of broad domains of HOX chromatin modifications that demarcate active and silent regions of the HOX loci (Bernstein *et al.*, 2005; Guenther *et al.*, 2005; Squazzo *et al.*, 2006). Actively transcribed domains of HOX are marked by RNA polymerase II occupancy and histone H3 lysine 4 dimethylation in the gene-coding and intergenic regions whereas transcriptionally silent domains are marked by histone H3 lysine 27 trimethylation and Polycomb group protein occupancy, such as the Polycomb group protein subunit Suz12 (Figure 2b). These chromatin domains are regulated in a site-specific manner. Moreover, a number of noncoding RNAs are transcribed in a site-specific manner (Sessa *et al.*, 2007).

In addition to normal homeostasis, a similar genomic approach may be employed to investigate diseases of the skin. The HOX loci are also important for epidermal appendage development and regulation of cell growth. HOXC13 is required for hair outgrowth (Godwin and Capecchi, 1998). Translocation of the human Trithorax protein gene MLL, leading to activation of the HOX loci, is a frequent etiology of leukemias (Guenther *et al.*, 2005), and the Polycomb group protein gene EZH2 is frequently amplified in human epithelial tumors and in melanoma (Bracken *et al.*, 2003). For instance, studies of genome-wide occupancy sites of MLL in leukemic cells have suggested that it functions as transcriptional start site-specific histone methyltransferase (Guenther *et al.*, 2005). With the easy accessibility of skin disease tissues and cells, interrogation with tiling microarrays is a promising tool to enhance our understanding of the epigenetic regulation of regulatory genes in skin and how these epigenetic codes may be disrupted during disease.

Tissue microarray: genomics and proteomics in the context of skin architecture

Gene and tiling microarrays are useful for elucidating the genetic and epigenetic elements that differentiate cell types across the body; however, it is equally important to determine the *in vivo* anatomic localization of genes in the three-dimensional context of the skin. To address this challenge, we have constructed a ''skin diversity'' tissue microarray, where multiple skin sections are placed on a single slide to be used for *in situ* hybridization and immunohistochemistry. Our tissue microarray is comprised of 42, two-millimeter formalin-fixed, paraffin-embedded cores of skin from diverse anatomic sites and 8 internal organs such as cervix, intestine, lung, liver and bone (Figure 3a). Immunohistochemistry or RNA *in situ* hybridization can be performed on all 50 tissues in parallel, allowing unbiased and high-throughput comparison of protein or gene expression levels and localization. A potential limitation of this technology is that proteins and mRNAs present in low levels may be better visualized in frozen sections than formalin-fixed tissues, and conditions for antigen retrieval or signal amplification may need to be developed to visualize low-abundance gene products.

To illustrate the use of such a skin diversity tissue microarray, we performed RNA *in situ* hybridizations for keratin 14 and keratin 9. As expected,

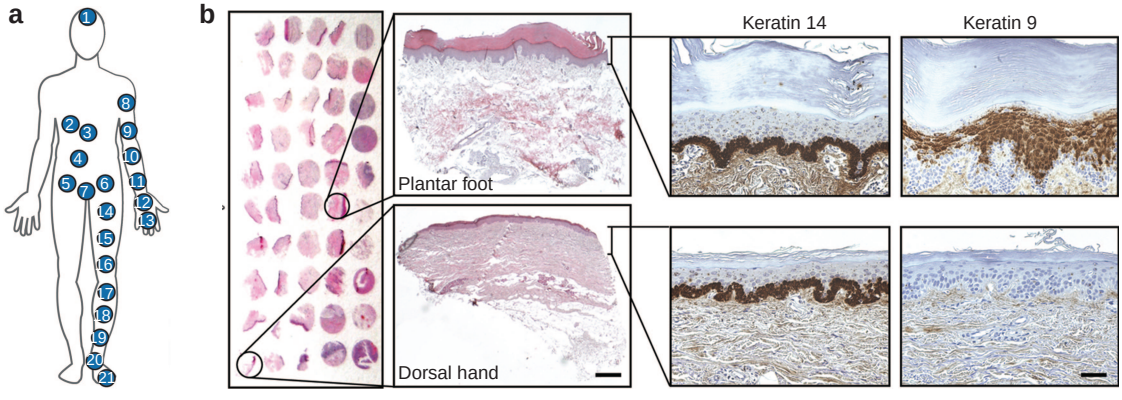

Figure 3. A human skin diversity tissue microarray. (**a**) Positional map of tissue microarray (left) comprised of 42, two-millimeter longitudinal cross-sections of skin and 8 sections of other organs, in total representing 21 unique sites of skin (blue circles). Hematoxylin and eosin staining of the tissue microarray (middle) and zoom in of cores representing plantar (top) and dorsal hand skin (bottom). Bar = 250 μm. (**b**) Skin section from plantar skin (top) and dorsal hand (bottom) following *in situ* hybridization with probes complimentary to either keratin 14 or keratin 9 mRNA. Bar = 50 μm.

we observed expression of keratin 14 in the basal layer of epidermis in skin from all anatomic sites. Keratin 9 is a suprabasal keratin of palmoplantar skin, and indeed only palmoplantar skin on our tissue microarray showed strong keratin 9 signal (Figure 3). The skin diversity tissue microarray should be useful for the discovery and validation of novel site-specific genes or signaling pathways. Again, this approach can be extended to include tissues of skin diseases to monitor the *in vivo* expression of genes perturbed in disease. The combined power of gene expression, tiling and tissue microarrays will greatly facilitate our understanding of the genes that are important in skin patterning and their roles in skin disease.

Conclusion and future challenges

The use of multiple systems biology approaches has started to paint a picture of the diversity of human dermal fibroblasts and the anatomic patterning of skin at the molecular level. This area of investigation is still at an early stage and much remains to be learned. Four challenges are likely to engross investigators in the near future. First, the transcriptional network of HOX genes in adult skin needs to be clarified. The mammalian targets of human HOX genes have eluded detection for many years owing to lack of human material and the embryonic lethality of most HOX genes in mice (Svingen and Tonissen, 2006). Primary human fibroblasts may be a tractable system to study the transcriptional network of HOX genes. Second, the epigenetic mechanisms that maintain site-specific gene expression programs remains incompletely understood. The identification of specific chromatin domains, their specific histone modifications, and associated noncoding RNAs are providing a list of candidate factors that may play a role in this regulatory program. Third, how these mechanisms of positional identity in normal skin may relate to the pathogenesis of many skin diseases with site-specific manifestations is largely unknown. Nonetheless, the history of investigative dermatology provides many examples of developmental pathways that become subverted and drive skin diseases, including diseases of epidermal adhesion, inflammation, and skin cancers. Fourth, site-specific differentiation is a dynamic process. For instance, hair cycling in many species occur in a seasonal fashion, and in mice hair cycling progresses gradually in an anterior–posterior fashion (Stenn and Paus, 2001). The use of conditional genetic approaches that can synchronize dynamic developmental programs by inducible arrest and release may aid to capture the temporal regulation of site-specific differentiation (Hutchin *et al.*, 2005; Sarin *et al.*, 2005; Estrach *et al.*, 2006). It is likely that a multifaceted approach incorporating emerging technologies will provide a wealth of information about the molecular cues involved in skin patterning and their dysregulation in skin diseases.

CONFLICT OF INTEREST

The authors state no conflict of interest.

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